

Characterization of Lactic Acid Bacteria Producing Bacteriocin from Lemuru (*Sardinella lemuru*) Smoked Fish Isolation

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Abstract. Bacteriocin is a ribosomal synthesized peptide which functions to inhibit the growth of pathogenic bacteria so that it can be used as a biopreservative. Many bacteriocins are produced by lactic acid bacteria. In some types of smoked fish found lactic acid bacteria found lactic acid bacteria that can produce bacteriocin.. This study aims to obtain lactic acid bacteria and test antibacterial from smoked fish type *Sardinella lemuru*.. Bacteria isolated from fish meat located on the head, body, and tail with agar media enrichment method MRS + CaCO₃. Colonies were identified based on morphological and physiological characters and antibacterial tests using the well method with MHA media. The isolation results had been obtained 11 isolates of lactic acid bacteria. One isolate potential is LM 2 had *E. coli* inhibition of 7.30 ± 0.6 mm, inhibition zone for *S. aureus* 7.0 ± 0.0 mm, and inhibition zone for *Salmonella* 16.3 ± 0.8 mm. The observation of the morphological, fisiological, biochemical characteristics of these isolates can be categorized into the *Lactobacillus plantarum*.

Keywords: antibacterial, smoked fish, *Sardinella lemuru*, lactic acid bacteria

1. Introduction

Nutrient content in fish consists of protein (6-24%), fat (0.2-2.2%), water (58-80%), and minerals (2.5-4.5%) [1]. According to [2]"fish have a water content of 76 g per 100 g of fresh fish weight". The presence of high water content is a suitable medium for the growth of spoilage bacteria or other microorganisms. Damage to fish also occurs during storage due to contamination of microorganisms from outside or in the food product. The process of spoilage in fish can be prevented by conducting proper processing such as by salting, fermentation, fumigation, use of radiation, and adding preservatives (preservation). Preservation is a way to preserve food products that aim to minimize the contamination of microorganisms. The use of preservatives has been proven to extend shelf life [3].

Preservative ingredients consist of two kinds, namely chemical and natural (bio-preservative). Illegal chemical preservatives used for food preservation such as formalin. The use of formalin can cause side effects on the human body, such as irritation, mutagenic, and carcinogenic. The use of formalin with high content in food can cause poisoning in the human body, such as pain in the stomach accompanied by vomiting, blood circulatory failure, and the onset of depression [4][5].

Based on the source, bio preservatives are grouped into 3 (three) which are derived from plants, animals and microorganisms. In plants contain compounds that can inhibit pathogenic microorganisms such as phenol compounds and their derivatives, terpenes and terpenoids, alkaloids, polypeptides and steroids. In animals the compounds contained are chitosan, lactoferrin, lysozyme, whereas those derived from microorganisms are the presence of organic acids and bacteriocin [6].

Bacteriocin is commonly found in Lactic Acid Bacteria (LAB) of the genus *Enterococcus* which produces an antimicrobial peptide named enterococcin. Bacteria of the genus *Enterococcus* are found in the intestines of animals. *Enterococcin* has stability in extreme conditions and is safe if applied to food products [7]; [8]; [9]; Vescovo et al., 2006 [10]). Other lactic acid bacteria found in the genus *Lactobacillus*, namely bacteriocin plantacin B as antimicrobial peptides are produced by *Lactobacillus plantarum* NCDO 1193, antimicrobial peptide Lactation F produced by *Lactobacillus acidophilus* 11088, antimicrobial peptide produced by *Lactobacillus plantarum* NCDO 1193, sakacin A antimicrobial peptide produced by *L. sake*[11] and pediocin antimicrobial peptide by *Pediococcus acidilactici* F-11 [12]. The mechanism of inhibiting antimicrobial substances can be done by disrupting the formation of cell walls, reacting with cell membranes, inactivating enzymes, and inactivating the function of genetic material.

Bacteriocin is produced by LAB, one of its sources is from fermented fish products and non-fermented fish products [13]; [14]). In research conducted by [14] about bacteriocin characterization from smoked salmon, obtained by lactic acid bacteria isolate *L. sakeii* which has the ability to produce bacteriocin. The bacteriocin obtained is one of the class IIa bacteriocins, sequencing G, has resistance characteristics at 30 °C and pH 5.5 and is able to inhibit *Escherichia coli* and *Pseudomonas fluorescens*.

2. Methodology

2.1 Study Area

Lactic acid bacteria were isolated from smoked lemuru fish (*Sardinella lemuru*.) Obtained from the Smokers' center of "Asap Indah" Demak, Indonesia. Test bacteria used for testing antimicrobial activities include *E. coli*, *S. aureus*, and *Salmonella*. The media used where the de Man Ragosa Sharpe Agar (MRSA), CaCO₃ NaCl, MRS broth, 70% alcohol, Gram Stein such as a crystal violet solution, legal solution, acetone alcohol and safranin. It also used emersion oil, 0.85% physiological saline solution, 3% hydrogen peroxide (H₂O₂) solution. The tools used are Erlenmeyer, test tubes, Bunsen, ose needles, cover glass, 0.25 µm milipore, centrifugator, centrifuge tubes, incubators, vortex, autoclaves, and VITEK-2 Biomerieux, USA.

2.2 Measurement of pH smoked fish

Lemuru smoked fish sample was weighed as much as 10 g, then cut into small pieces and then crushed using a mortar. Samples were put into 20 ml of distilled water and let stand for 1 minute. Furthermore, it is poured into a glass beaker as much as 10 ml, then the pH is measured using pH meter. Before the pH meter is used the sensitivity of the needle must be tested with a buffer solution of pH 7 and pH 4. The amount of pH is the reading of the pH needle after the constant scale needle is in its position [15].

2.3 Measurement of Smoked Fish Water Content

Porcelain dishes and covers are dried in an oven 105-110 °C for 1 (one) hour. Then cooled in a desiccator for 30 minutes and weighed (A g). The sample was weighed as much as 2 g and placed in a porcelain cup of known weight (B g). then dried in an oven at 105-110 °C until the sample reaches a constant weight for 24 hours. Then the sample is cooled in a desiccator for 30 minutes and weighed (C g). This balance is repeated until a constant weight is obtained. The percentage of water content is calculated using the following formula [15]

$$\text{Water content} = \frac{(B - C)}{(B - A)} \times 100\%$$

Where: A = cup dry weight (gram); B = dry weight of the cup and initial sample (grams); C = the dry weight of the cup and sample after drying (grams).

2.4 Isolation of Lactic Acid Bacteria

A sample of 5 g was mashed using a mortar and then put into 45 ml medium MRS broth + CaCO₃ than homogeneous. Subsequently incubated in a shaker incubator for 72 hours at a temperature of 30° C, with a speed of 150 rpm. The results of the incubation were 7 (seven) dilutions, from each dilution 1 (one) ml was taken. In the 105-107 dilution series 1 (one) ml is taken and put in a petri dish. MRS agar + CaCO₃ media is poured into a

petri dish which has been filled with dilution solution. Furthermore, it was incubated at 37°C for 72 hours. Bacterial colonies are taken from colonies that form clear zones. Next, it is etched on MRSA medium to obtain pure colonies [16].

2.5 Gram Staining

Bacteria were inoculated in MRS agar and incubated for 48 hours at 37 ° C. Bacterial colonies are taken 1 (one) ose needle and placed on glass objects that have been given distilled water. The bacterial suspension is mixed and flattened an area of ± 1 cm². The bacterial suspension on the class of the object is air dried and then fixed over the Bunsen flame. After a cold bacterial suspension, drops of Hacker's crystal violet (Gram A) are 2-3 drops and allowed to stand for 1 minute. Then washed with running water, then dried. The next step is dripping with mordant solutions (Gram B) and left for 1 minute. Then washed with running water and dried. Furthermore, aqueous solution (Gram C) is dropped for ± 30 seconds, then washed with running water and dried. The last step is dripping with safranin (Gram D) solution and left for 2 minutes, then washed with running water and dried. The results of painting are then observed under a microscope. Cells that show blue color are Gram-positive, and cell bacteria while red color is negative [17].

2.6 Catalase Test

Colony of bacteria is taken as much as 1 is and etched on a glass object. Then drop with hydrogen peroxide (H₂O₂) 3%. The suspension is mixed slowly. The change is observed, if the positive, catalase is marked by the formation of air bubbles. [18].

2.7 Morphology Testing Colony

Bacterial isolates were inoculated on MRSA in a petri dish. Furthermore, it was incubated for 48 hours at 37 ° C. Growing colonies were observed and identified the shape of the colony, elevation, edge shape [19].

2.8 A Test antimicrobial activity using the wells method againts *Salmonella* sp. 29213, *S. aureus*14028, and *E. coli*

Test bacteria were grown on nutrient agar (NA) medium and subsequently incubated for 24 hours at 37 ° C. Colonies of growing bacteria were taken with a dose, needle and then put into a 0.85% physiological NaCl solution. The suspension was also synchronized with McFarland 3. The test bacteria were taken by means of a swab using sterile cotton over Mueller Hinton Media. In order to leave it for a while, then make a well using a Durham tube.

The supernatant yield of bacteriocin extract was uniformly uniform with the McFarland 3 standard. The concentration of McFarland 3 was 9 x 10⁸ (a mixture of 0.5 ml of 1% BaCl₂ solution with 9.5 ml of H₂SO₄ 1%). 50 µl of bacterial supernatant was taken into these wells, then

incubated at 37°C for 24 hours. Inhibited zones formed were observed and measured in diameter [20].

2.9 Bacteria Identification Using Vitex-2 Compact Card

Used bacterial isolates aged 18-24 hours. isolates were grown in Mac Conkey media for aerobes and blood agar media for anaerobes. Next gram painting is done, which aims to find out the type of card used. if Gram is positive, identify using a GP card, AST GP. If the Negative Gram uses the GN card and the GN AST.

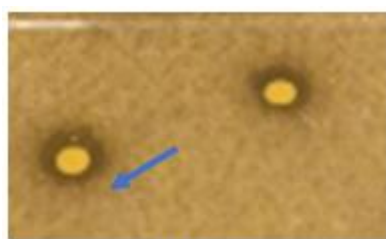
3. Result and Discussion

3.1 Water Content and pH of Smoked Fish

The results of the analysis of water content in flying smoked fish with 3 replications amounted to 57.93% and has a pH of 6. Based on SNI (01-2356-1991) smoked fish the maximum water content contained in the product is 60%. The water content of smoked fish is in accordance with SNI standards. Moisture content that is in accordance with these standards can be caused by times in smoking, the amount of fuel used during smoking, and the temperature of smoking is appropriate. However, if in the fumigation of fuel use and the length of fumigation time is not right, it will affect the water content in smoked fish. This is because the longer the smoking time and a lot of fumigation fuel, the temperature in the smoking room will increase, and the temperature of the smoking room fluctuates. A low pH value can affect the shelf life of the product. The smaller the pH of the fish, the longer the shelf life will be. The low pH value due to smoking due to the use of fuels containing organic materials that cause pH to fall.

3.2 Isolation of Lactic Acid Bacteria

The results of purification from isolation on the media MRSA + CaCO₃ obtained BAL isolates were identified as many as 15 isolates in each smoked fish sample with almost the same colony size and showed a clear zone around the colony. Clear zone formed around the colony due to CaCO₃'s neutralization of acids produced by lactic acid bacteria [21]. The presence of a clear zone in the MRS agar medium which was added by CaCO₃ indicates the production of acid in the bacteria produced by the isolate. Isolates that produce acids are thought to be lactic acid bacteria. Following one of the isolates that produce acid is presented in Figure 1.



Clear zone

Figure 1: Colony of Lactic Acid Bacteria

3.3 Morphology colony

The colony morphology, identification was carried out aiming to find out the shape, edges, elevation, and color of the colony. According to [19] that the observation of the shape of the colony is seen from above, the edge of the colony is seen from above; the elevation is seen from the side. From the test results obtained 15 colonies have a form of a circular colony with embossed edges, slippery colony edges, elevation of embossed shapes and the color of milk-white colonies. In a study conducted by [22] stated that bacteria that have Gram positive characteristics, do not form spores, have cocci or bacilli, and are catalase negative as indicated by the class of lactic acid bacteria.

Table 1: Table of Colony Morphological Test Results

Isolates Code	Colony Shape	Colony Edge	Elevation	Colony Color	Gram Staining	Cell Shape
LM 1	Round with raised edges	slippery	arise	Milk white	-	bacil
LM 2	Round with raised edges	slippery	arise	Milk white	+	bacil
LM 3	Round with raised edges	slippery	arise	Milk white	+	bacil
LM 4	Round with raised edges	slippery	arise	Milk white	-	bacil
LM 5	Round with raised edges	slippery	arise	Milk white	-	bacil
LM 6	Round with raised edges	slippery	arise	Milk white	+	bacil
LM 7	Round with raised edges	slippery	arise	Milk white	+	bacil
LM 8	Round with raised edges	slippery	arise	Milk white	-	bacil
LM 9	Round with raised edges	slippery	arise	Milk white	+	bacil
LM 10	Round with raised edges	slippery	arise	Milk white	+	bacil
LM 11	Round with raised edges	slippery	arise	Milk white	+	bacil
LM 12	Round with raised edges	slippery	arise	Milk white	+	bacil
LM 13	Round with raised edges	slippery	arise	Milk white	+	bacil
LM 14	Round with raised edges	slippery	arise	Milk white	+	bacil
LM 15	Round with raised edges	slippery	arise	Milk white	+	bacil

3.4 Catalase test

Catalase testing is done by dripping a solution of hydrogen peroxide (H₂O₂) 3% in isolates that are 48 hours old. The positive reaction if CO₂ bubbles appear. The results of this catalase test were 15 isolates from smoked fish. This test is carried out to determine the ability of bacteria to produce the enzyme catalase and tolerance to oxygen. Catalase enzyme is an enzyme that is able to catalyze the direct conversion of hydrogen peroxide (H₂O₂) which is toxic to bacterial cells into water and oxygen. Lactic acid genus *Lactobacillus* is a group of bacteria that do not have the enzyme catalase, but have the enzyme peroxidase to convert H₂O₂ which is toxic into H₂O. The following reaction equation from the catalase test:



3.5 Gram Staining

Gram's staining aims to determine cell morphology and types of bacteria. On Gram staining, Gram positive bacteria will be purple / violet while Gram negative bacteria are red. The results of the Gram staining obtained 11 isolates from Lemuru (*Sardinella lemuru*) smoked fish were Gram positive with the form of bacillus cells (stem) and 4 isolates were Gram negative with the form of bacillus cells (stem). The image of Gram stain can be seen in Figure 2.

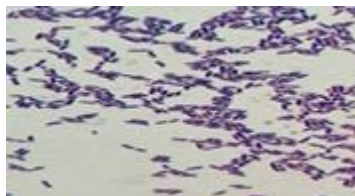


Figure 2: Gram Positif Isolat LM 2

Determination of Gram staining based on differences in bacterial cell wall structure. The peptidoglycan layers contained in the cell walls of Gram-positive bacteria are thicker than Gram-negative bacteria. Gram positive bacteria contain teichoic elements as much as 50% of the dry weight of the cell wall. This element serves to maintain ion transport, cell wall integrity, choline replacement by ethanolamine so that it is resistant to autolysis and maintain external permeability [23].

In Gram staining, Gram positive bacteria are purple because these bacteria have more lipid content so that the bacterial cell walls will be more easily hydrated due to treatment with alcohol. Hydrated cell walls cause cell pores to become smaller and their permeability to be reduced. Violet crystals, which are the main color, cannot get out of the cell and the cells will remain purple. While

the negative Gram will be red because the bacteria lose the color of violet crystals when repressing with alcohol, but are able to absorb a counter dye namely safranin. Gram-negative bacteria have a higher percentage of lipids, fats, or substances such as fat than those contained in gram-positive bacteria. The walls of Gram negative bacterial cells are also thinner than Gram positive bacterial cells [19].

3.6 Antimicrobial Activity Test Using the Wells Diffused Method

The media used in this test are Muller Hinton Agar (MHA) Media which contains indicator bacteria (*S. aureus*, *E. coli*, and *Salmonella*). The inhibition zone diameter produced in the wellness area was measured using a caliper after incubating for 24 hours at 37 ° C. Observation of inhibitory activity aims to determine the ability of bactericide to inhibit pathogenic bacteria. The greater the inhibitory power, the clearer zones that are formed become wider [24], [14]. Based on research conducted by [25], inhibition zones with inhibition zones of > 5 mm were categorized as small, inhibitory zones of 5-10 mm were categorized as a medium, and inhibitory zones of > 10 mm were categorized as high.

The results of bacteriocin testing on pathogenic bacteria showed the presence of clear zones and turbid zones. The clear zone formed can be interpreted that the isolates tested have inhibitory activity due to bacteriocin activity while the turbid zone is indicated that pathogenic bacteria are resistant to bacteriocin so that bacteriocin is no longer able to inhibit the activity of pathogenic bacteria. This could be due to an unbalanced bacteriocin concentration with pathogenic bacteria, incubation time, and the type of pathogenic bacteria used [26].

Based on the results of the activity test, it was found that all isolates formed inhibition zones against 3 (three) test bacteria, namely *E. coli*, *S. aureus*, and *Salmonella*. with different inhibition zones. There is 1 (one) isolate which is potentially as a bacteriocin producing which can inhibit 3 (three) test bacteria. The isolate is LM 2. With the largest inhibition zone inhibiting *Salmonella* by 16.3 mm which is included in the high category. For more details, see Table 2.

According to [27] the difference in inhibitory activity due to bacteriocin has inhibitory activity against specific bacteria and has a close kinship with the bacteriocin-producing bacteria. Inhibitory activity is also influenced by differences in the structure of inhibited bacterial cell walls that affect the resistance of a bacterium to antimicrobial substances

Table 2: Bacteriocin Activity Table Against Test Bacteria

Isolate Code	Positive Control	Inhibitory zone diameter against test bacteria		
		<i>E.coli</i> (mm)	<i>S. aureus</i> (mm)	<i>Salmonella</i> (mm)
LM 1	13,0±4.2	7,00±1,0	8,6±1,7	9,6±1,7
LM 2	7,5±0.70	7,3±0,6	7,0±0,0	16,3±0,8
LM 3	26,5±9,1	7,30±0,6	11,0±3,6	10,3±4,0
LM 4	9,5±0,7	7,00±0,0	8,6±0,6	7,6±1,5
LM 5	7,5±0,7	7,6±1,2	9,0±1,0	12,3±0,67
LM 6	28,5±4,9	8,6±1,5	9,6±2,1	9,6±0,6
LM 7	10,5±0,7	7,6±1,2	9,3±0,6	11,3±1,2
LM 8	9,0±1,4	8,00±1,0	8,7±0,6	11,7±0,7
LM 9	14,0±1,4	7,30±0,6	8,6±0,6	9,6±3,6
LM 10	23,0±2,8	7,6±0,6	9,0±1,0	11,3±3,2
LM 11	7,0±0,0	7,0±0,0	7,0±0,0	10,6±0,4
LM 12	25±7,7	6,70±1,0	8,0±1,7	10,3±4,0
LM 13	8,0±1,4	8,3±2,0	7,6±1,0	9,0±1,0
LM 14	8,0±0,0	8,00±1,0	6,0±1,0	8,6±0,6
LM 15	8,0±0,0	7,7±2,1	9,7±0,7	7,3±0,7

Identification Using Vitek-2

Bacteria that will be identified are selected bacteria that have the greatest antibacterial activity. The bacteria selected in this study were bacterial isolates that had LM 2 codes. The results of identification of bacteria using Vitek-2 revealed that the LM 2 code isolate was *Lactobacillus plantarum* with a similarity of 96%.

4. Conclusion

A total of 15 isolates were isolated from lemuru (*Sardinella lemuru*) smoked fish. The isolate had 11 were assumed lactic acid bacteria because had catalase negatif, Gram positive, had cell morphology are bacil. Isolate which has potential as a bacteriocin producer, is isolate LM 2 which effectively inhibits *Salmonella* bacteria by 16.3 ± 0.4 mm. Identification using vitek 2 revealed that LM 2 isolates were identified as *Lactobacillus plantarum* with 97% similarity.

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